

AD_____

Award Number: DAMD17-03-C-0088

TITLE: STRUCTURE-BASED DESIGN OF INHIBITORS TO THE CYTOTOXIN
RICIN

PRINCIPAL INVESTIGATOR: Jon Robertus, Ph.D.

CONTRACTING ORGANIZATION: University of Texas at Austin
Austin TX 78712-1500

REPORT DATE: April 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01-04-2007		2. REPORT TYPE Final		3. DATES COVERED (From - To) 15 Jun 03 – 14 Dec 06	
4. TITLE AND SUBTITLE STRUCTURE-BASED DESIGN OF INHIBITORS TO THE CYTOTOXIN RICIN				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER DAMD17-03-C-0088	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Jon Robertus, Ph.D. E-Mail: jrobertus@mail.utexas.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Texas at Austin Austin TX 78712-1500				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT: Ricin is a cytotoxin and a known bioterrorist weapon. The Army is pursuing anti-ricin vaccines, but plans to develop an efficacious antidote to the toxin, for cases where vaccination is not appropriate. The goal of this project is use the X-ray structure of ricin A chain (RTA) as a template for inhibitor design. Computer modeling and X-ray screening aid in the design process. Inhibitors which bind to the RTA substrate specificity site have been identified. A platform, 9-deazaguanine, has been shown to bind in the RTA active site and act as a weak inhibitor. However, efforts to derivatize and diversify the platform via triazole "click" chemistry have met with unanticipated difficulties. None of the new compounds exhibits greatly improved inhibitory properties. A virtual screen of available compounds suggests catechols may provide a novel platform for future work.					
15. SUBJECT TERMS ricin; inhibitor design; X-ray structure; novel compounds					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	12	19b. TELEPHONE NUMBER (include area code)

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5
Task 1.....	5
Task 2.....	6
Task 3.....	6
Task 5.....	9
Key Research Accomplishments.....	10
Reportable Outcomes.....	10
Conclusions.....	10
References.....	12
Appendices.....	

INTRODUCTION

This contracted project is aimed at designing, synthesizing, and testing small molecule inhibitors of the cytotoxin ricin. Ricin is a class B biological agent which is known to be in the possession of terrorist groups (Loyd, 2001). Although not as menacing as infectious agents, ricin is of great concern because of the ease with which large amounts of semi pure material can be produced (Wellner et al, 1995). The Army is proceeding with vaccine development for key military personnel (Olson et al, 2004). However, wide spread vaccination of the military or civilian population is not practical or desirable, and so there is a need for an efficacious antidote. In addition to its utility such a compound could reduce panic that arises from a relatively minor terrorist incident. Our area of expertise is the rational design of inhibitors of enzymes like ricin A chain, RTA (Yan et al, 1997; Miller et al, 2002). We have elucidated the three-dimensional structure of ricin and this model serves as a template for the design of small molecules that can bind tightly and inactivate the toxin. These inhibitor compounds should also incorporate elements of drug design, including solubility, stability, and low toxicity. We have used computer searches to identify classes of inhibitors that act as “platform” molecules. These platforms have been modified and appended creating novel inhibitors for RTA. This particular project is a collaboration between structural biologists (Robertus group) and synthetic chemists (Kerwin group) to extend our previous research efforts on antidotes. It is a step-wise process, beginning with modest inhibitors, which are then sequentially improved after analysis, to produce ever more potent compounds; the program is scheduled to last three years. Our overall goal is to create a ricin inhibitor which is efficacious at inhibiting ricin intoxication of cultured cells, and itself is non-toxic.

BODY

The original Statement of Work (SOW) is as follows:

Task 1: Design improved specificity pocket (months 1 - 12)

- a. Prepare 9-oxaguaninine
- b. Prepare other related heterocycles

Task 2: Identify ligands for second ricin binding pocket (months 1 - 18)

- a. Use computer searches based on ricin structure
- b. Apply crystallographic screening of shape-diverse sets of commercial compounds

Task 3: Prepare tripartite inhibitors joining best specificity pocket and second pocket moieties with appropriate linkers (months 9-24).

Task 4: Use iterative crystallographic algorithm to refine tripartite inhibitors (months 12-36)

- a. Modify compound shape to fit enzyme contours
- b. Design compounds for maximum water solubility and biological uptake

Task 5: Test biological efficacy of inhibitors as ricin antidotes (months 1-36)

- a. Candidate inhibitors will be tested against ricin enzyme activity
- b. Ricin inhibitors will be tested for protective action in cultured human lung cells
- c. The most promising ricin antidotes will be sent to a commercial testing facility for initial human safety tests using a panel of enzyme and receptor assays.

ACCOMPLISHMENTS FOR (CALENDAR) YEAR 4

TASK 1: Design improved specificity pocket ligands

Our search for improved specificity pocket ligands, based on novel synthesis, has stopped. However, some new methods, which were not available to us when this proposal was first written, have become available. In particular, we have acquired a 16 node parallel computer with 32 dual processors. We also acquired three pieces of virtual drug screening software, called GOLD (Jones et al, 1997; Verdonk et al, 2003), eHiTS (Zsoldos et al, 2006), and Surflex (Jain, 2003). These programs were validated by docking a short list of known RTA inhibitors (Miller et al, 2002); this test showed that the known inhibitor pterioic acid (PTA) was correctly docked, compared to the X-ray structure, by all three programs. Next, in a test using 1000 random compounds from the Sigma Aldrich data base (Irwin & Shoichet, 2005), PTA was ranked in the top 5% of predicted binding strengths by each of the three programs. This suggested that the programs were able to recognize the characteristics of a true inhibitor. In addition to confirming that PTA was an RTA inhibitor, the virtual screen produced some novel candidate compounds as inhibitors. Appropriate to this task is the identification of potential new platform molecules. The program GOLD predicted that a compound called Dobutamine would be an inhibitor. We tested it in a ribosome based protein synthesis assay and estimated the IC_{50} at 1 μ M (Figure 1). Poor solubility precludes an accurate measure, but if this value holds up in subsequent analysis, it will be about 500 times more potent than any previously identified small molecule inhibitor of RTA. Figure 2 shows the predicted binding of the compound, although we have been unable to form crystals of this complex to confirm the binding mode. What is interesting is that it suggests that catechols may serve as a specificity pocket platform.

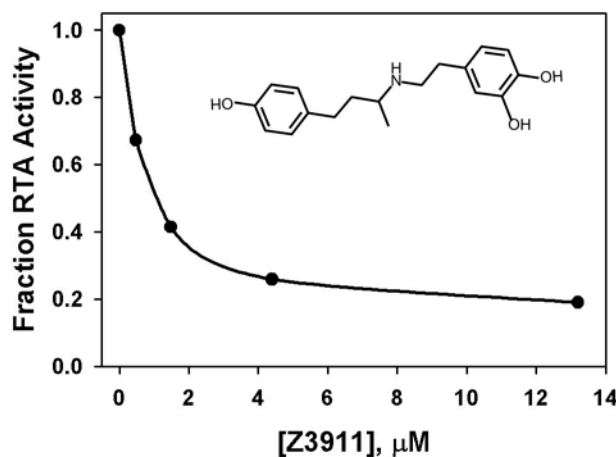


Figure 1 Dobutamine is a strong inhibitor of RTA. The curve shows an IC_{50} of $1 \mu\text{M}$; the structure of Z3911 is also indicated.

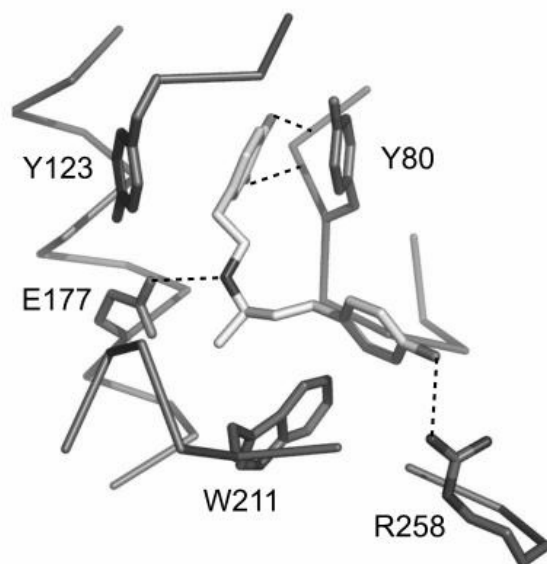


Figure 2 Predicted binding of dobutamine by GOLD. The inhibitor is shown in light bonds, and elements of the RTA active site are darker. Hydrogen bonds are dashed lines; the catechol head group hydrogen bonds to the backbone of V81.

TASK 2: Identify ligands for second ricin binding pocket

We reported last year that all efforts to identify ligands binding to the “second pocket” adjoining the RTA specificity site (Monzingo & Robertus, 1992) by X-ray diffraction have failed. A major problem appears to be that most of the candidates have poor solubility. We know that the K_d values for specific active site molecules, like adenine and pterins, are 1 -2 mM. It is likely the K_d for this other site, when taken alone, is at least that high. The screening of shape diverse libraries requires a good deal of organic solvent to dissolve the panel of compounds and this has interfered with crystal screening, as it does with kinetic screening. It may be that virtual screening will be useful in identifying ligands for this second site; indeed docking programs suggest that very nonpolar groups like naphthalene, fluorene, and acridan bind deep in this second pocket. However, their poor solubility makes this difficult to confirm and bi and tri partite synthesis is not yet in a position to incorporate these groups.

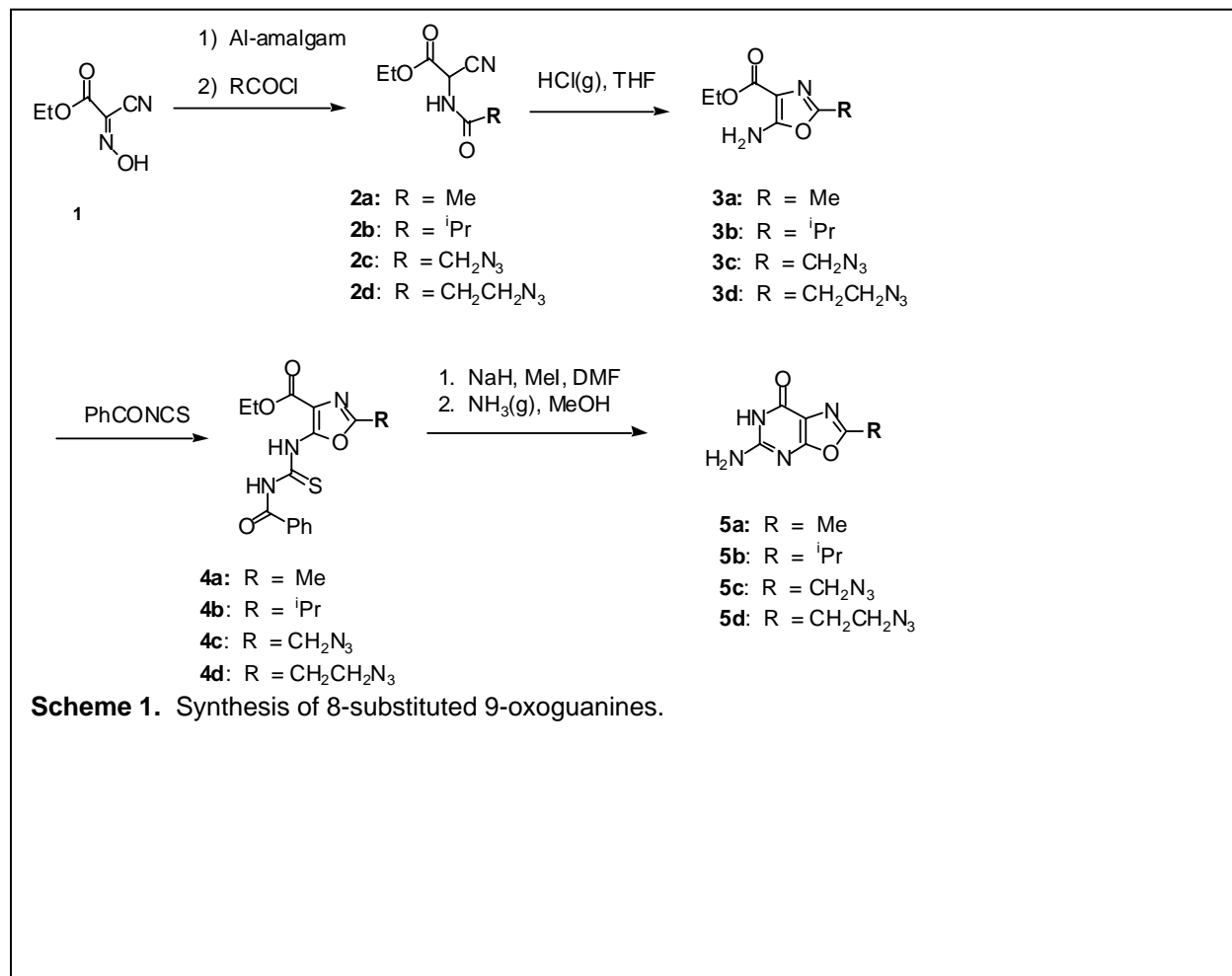
TASK 3: Prepare tripartite inhibitors joining best specificity pocket and second pocket moieties with appropriate linkers (months 9-24).

Synthetic approaches to tripartite inhibitors have focused on elaborating two specificity pocket-binding scaffolds, 9-oxoguanine and 9-deazaguanine. Although progress has been made in developing synthetic approaches, no potent inhibitors have been identified to date.

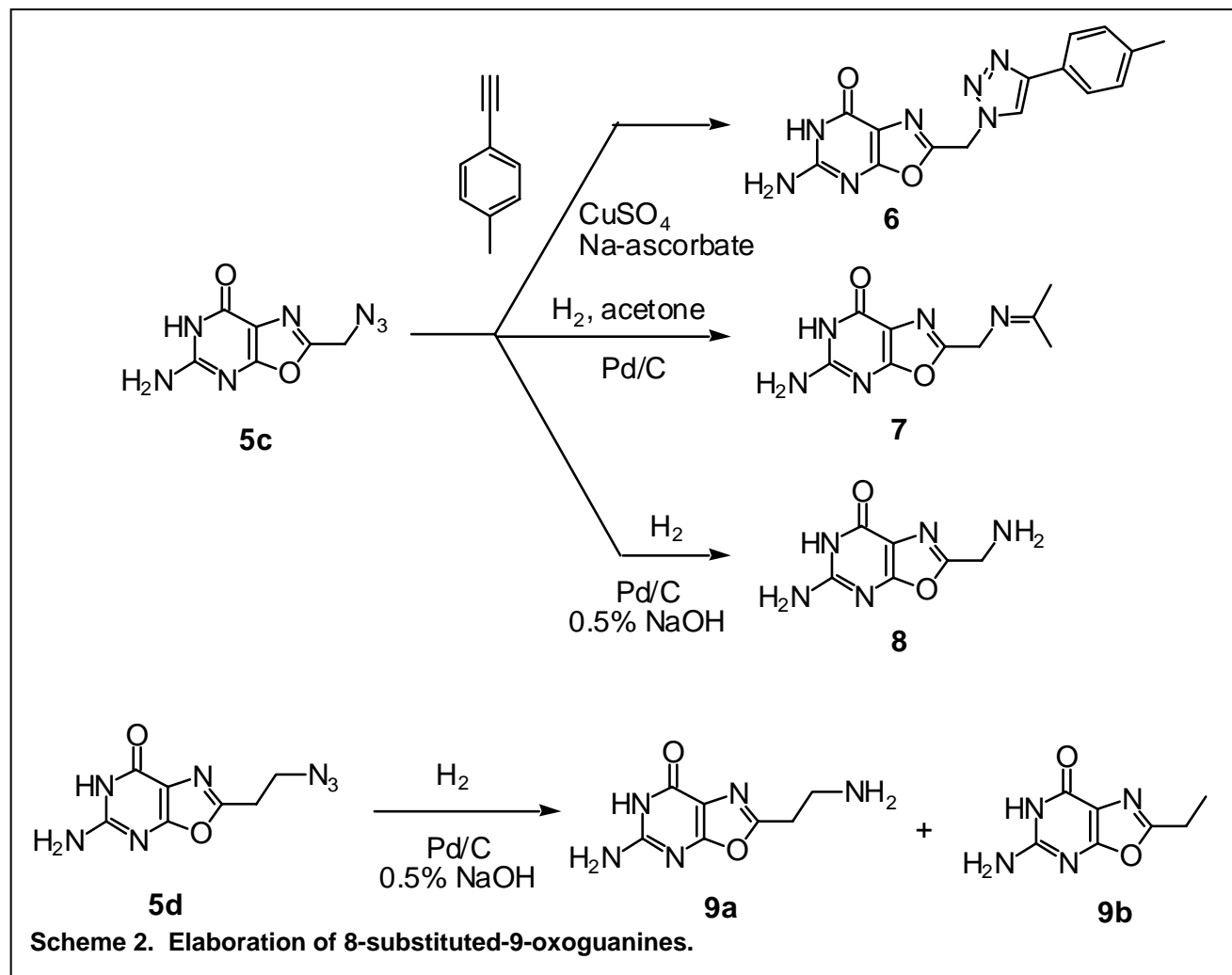
A. Prepare elaborated 9-oxaguanine analogues

Work continues on constructing inhibitors based on the 8-methyl-9-oxa-guanine scaffold. During the previous reporting period, a general route to 8-substituted 9-oxo-guanines was

found. This route (Scheme 1) requires elaboration of appropriate 2-substituted 5-amino-oxazoles, which are then acylated with benzoyl isocyanate, activated by methylation, and cyclized in the presence of ammonia. Although this route is somewhat lengthy, it has been used to successfully produce a number of these 8-substituted 9-oxo-guanines, as shown in Scheme 1.



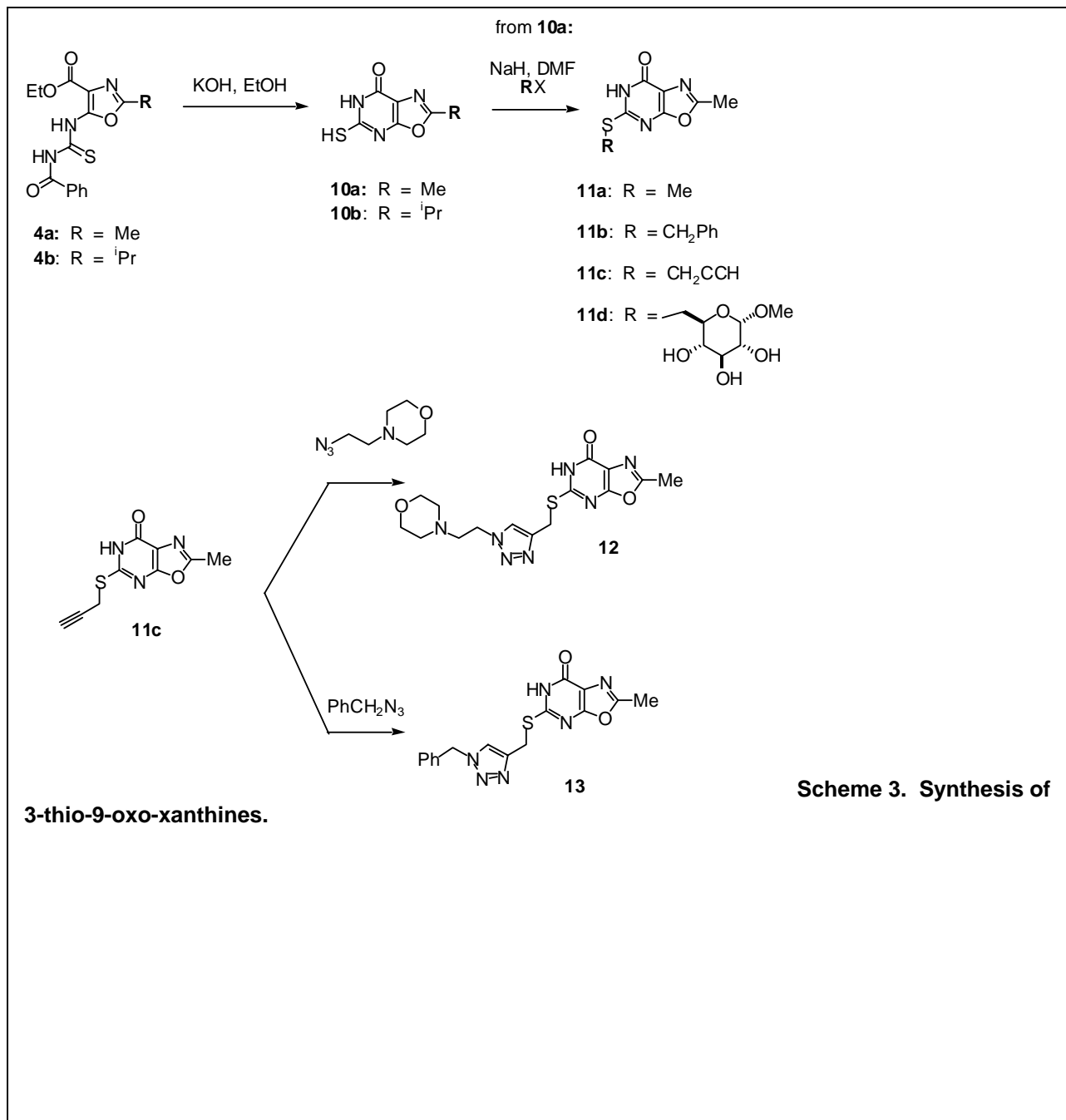
Given the length of the syntheses of these 8-substituted-9-oxo-guanines (Scheme 1), a method of introducing more elaborate 8-substituents from a common 8-substituted precursor



was sought. In particular, the azido compounds **5c** and **5d** could serve as versatile intermediates by reduction to the amines, direct conversion to imines via the Staudinger reaction, or by “Click” chemistry involving coupling with terminal acetylenes to afford triazoles. We have preliminary results to support the viability of this approach, as shown in Scheme 2. The azidomethyl-substituted 9-oxo-guanine **5c** undergoes “Click” chemistry to afford **6**, can be reduced to the amine **8** or the imine **7**. A technical difficulty that had to be addressed in the latter two transformation is the limited solubility of **5c** in non-aqueous solvents. Under optimized conditions for the catalytic reduction of **5c**, the reaction is carried out in dilute aqueous NaOH. The azido-ethyl analog **5d** can also undergo reduction under these conditions; however, in this case a significant amount of the 8-ethyl analog **9b** is also obtained. This format hydrogenolysis of the azido group of **5d** presumably occurs via elimination to the 8-vinyl analog under the basic conditions required to solubilize **5d**, followed by hydrogenation to **9b**.

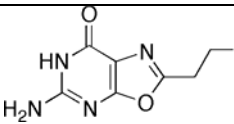
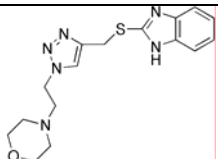
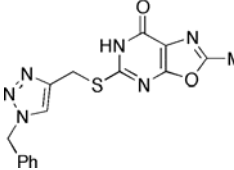
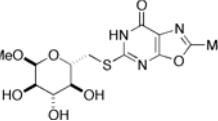
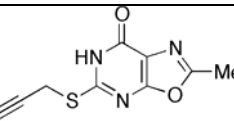
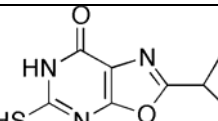
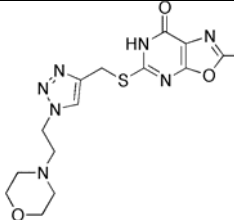
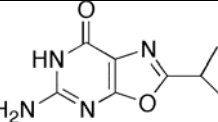
During the previous reporting period, we had noted that certain 9-oxo-3-thioxanthines related to **5a** displayed modest activity against ricin (Scheme 3). The *S*-methyl derivative **11a** was shown to have an IC_{50} of ~ 4.8 mM. In order to determine if this activity could be increased by modification of the 3- or 8-substituents, additional analogs were prepared, as shown in

Scheme 3. Cyclization of the 2-methyl or 2-iso-propyl oxazoles **4a,b** in base affords the 8-substituted 3-thio-9-oxo-xanthines **10a,b**. Alkylation of the 8-methyl derivative **10a** proceeded well with a variety of electrophiles to afford the S-alkylated derivatives **11a-d**. We once again took advantage of the versatility of Click chemistry to further elaborate the propargyl derivative **11c** to afford the triazoles **12** and **13**.



Task 5: Test biological efficacy of inhibitors as ricin antidotes (months I-36)
a. Candidate inhibitors will be tested against ricin enzyme activity

Over the past year we have tested the inhibition of RTA by a number of novel compounds. This is summarized in Table 2. The inability to create useful pendants from the active site platform molecules (purines or pterins), has limited our progress. The structural designs for larger bi and tri partite inhibitors may have merit, but unanticipated and continuing difficulties in their synthesis and the insolubility of the few tripartite inhibitors that were prepared have prevented us from testing these candidates. The list below is largely composed of intermediate compounds in a more elaborate synthetic strategy.

Compound	ID	IC ₅₀	Compound	ID	IC ₅₀
	8-amino-9oxoG	Not detectable		RCN124	Not detectable
	RCN116	Not detectable		RCN126	Not detectable
	RCN118	Not detectable		RCN127	2.5 mM
	RCN120	1.6 mM		RCN128	2.8 mM

KEY RESEARCH ACCOMPLISHMENTS

Our virtual screening efforts suggest that a useful new specificity site platform may be catechols. Although no improved ricin inhibitors have been prepared by elaboration of our previously disclosed platform inhibitors, two key synthetic accomplishments have been met:

- Construction and elaboration of 9-oxo-3-thioxanthine [5-mercapto-6H-oxazolo[5,4-d]pyrimidin-7-one], including application of “click” chemistry.
- Construction and elaboration of 8-substituted-9-oxo-guanines [5-amino-6H-oxazolo[5,4-d]pyrimidin-7-ones].

REPORTABLE OUTCOMES

A manuscript describing the new synthetic route to 9-oxoguanine derivatives is in preparation.

CONCLUSIONS

The proposed inhibitor design project is far more difficult than originally envisioned. The active site of RTA is large and polar, evolved to accommodate a large number of weak interactions from a large RNA. Finding small molecules that make strong and specific interactions to compete with this large scale substrate binding is taxing. The 9DG platform molecule is an inhibitor, as are the 7-methyl and 7-propargyl groups. All can be seen in complex by X-ray. However, the “Click” chemistry-derived triazole constructs of 9-deaza guanine appear to be problematic - the large triazole group may prevent binding – and all of these analogs fail to inhibit. The 9-OG platform molecule is also an inhibitor, and synthetic difficulties in preparing more elaborate substituted 9-OG analogs have recently been overcome; however, none of these elaborated 9-OG inhibit better than the original platform. Even so, the diversity of subsites within the RTA target suggest that it should be possible to create tight binding inhibitors – it will simply require very skillful chemistry. We are also reasonably sanguine about the utility of *in silico* virtual screening. Tests recently completed suggest that the latest algorithms have merit, and may lead to identification of novel platform compounds and point to useful pendant groups as well.

“So what?”: The elaboration of two different platform inhibitors has as yet failed to lead to improved ricin inhibitors. The number and nature of the analogs prepared has been limited due to unforeseen problems with the synthetic routes and the very limited solubility of some of these elaborated compounds. Even after working around these problems, of the several new compounds that were prepared and tested, few were inhibitors and none were superior to the starting platform inhibitors. Our most recent approach has relied on “click” chemistry to link potential pendants to platforms. Although the chemistry is facile, the nature of the triazole linkers may not be optimum for ricin inhibition. None-the-less, it is essential to link larger pendants to the largely optimized platforms in order to improve binding by several logs. The route forward must carefully examine not only the nature of the pendant groups but also the linkers. In practical terms, this may require alternative platforms for which synthesis and solubility issues are more optimal for the evaluation of a large numbers of potential inhibitors.

REFERENCES

- Irwin, J. J. and Shoichet, B. K. (2005). "ZINC--a free database of commercially available compounds for virtual screening." J Chem Inf Model **45**(1): 177-182.
- Jain, A. N. (2003). "Surflex: fully automatic flexible molecular docking using a molecular similarity-based search engine." J Med Chem **46**(4): 499-511.
- Jones, G., Willett, P., Glen, R. C., Leach, A. R. and Taylor, R. (1997). "Development and validation of a genetic algorithm for flexible docking." J Mol Biol **267**(3): 727-748.
- Loyd, A. a. F., M. (2001). Bin Laden's poison manual. London Times. London, England.
- Miller, D. J., Ravikumar, K., Shen, H., Suh, J. K., Kerwin, S. M. and Robertus, J. D. (2002). "Structure-based design and characterization of novel platforms for ricin and shiga toxin inhibition." J Med Chem **45**(1): 90-98.
- Monzingo, A. F. and Robertus, J. D. (1992). "X-ray analysis of substrate analogs in the ricin A-chain active site." J Mol Biol **227**(4): 1136-1145.
- Olson, M. A., Carra, J. H., Roxas-Duncan, V., Wannemacher, R. W., Smith, L. A. and Millard, C. B. (2004). "Finding a new vaccine in the ricin protein fold." Protein Eng Des Sel **17**(4): 391-397.
- Verdonk, M. L., Cole, J. C., Hartshorn, M. J., Murray, C. W. and Taylor, R. D. (2003). "Improved protein-ligand docking using GOLD." Proteins **52**(4): 609-623.
- Wellner, R. B., Hewetson, J. F. and Poli, M. A. (1995). "Ricin: Mechanism of action, detection, and intoxication." J.Toxicol, Toxin Rev **14**: 483-522.
- Yan, X., Hollis, T., Svinth, M., Day, P., Monzingo, A. F., Milne, G. W. and Robertus, J. D. (1997). "Structure-based identification of a ricin inhibitor." J Mol Biol **266**(5): 1043-1049.
- Zsoldos, Z., Reid, D., Simon, A., Sadjad, S. B. and Johnson, A. P. (2006). "eHiTS: A new fast, exhaustive flexible ligand docking system." Journal of Molecular Graphics and Modelling **in press**.